# Type II kinase inhibitors targeting Cys-gatekeeper kinases display orthogonality with wild type and Ala/Gly-gatekeeper kinases

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**Abstract.** Analog-sensitive (AS) kinases contain large to small mutations in the gatekeeper position rendering them susceptible to inhibition with bulky analogs of pyrazolopyrimidine-based Src kinase inhibitors (*e.g.* PP1). This 'bump-hole' method has been utilized for at least 85 of ~520 kinases, but many kinases are intolerant to this approach. To expand the scope of AS-kinase technology, we designed type II kinase inhibitors, **ASDO2/6** (**A**nalog-**S**ensitive '**D**FG-**O**ut' kinase inhibitors-**2**/**6**), that target the 'DFG-out' conformation of cysteine (Cys)-gatekeeper kinases with submicromolar potency. We validated this system *in vitro* against Greatwall kinase (GWL), Aurora-A kinase and Cyclin-dependent kinase-1 and in cells using M110C-GWL expressing mouse embryonic fibroblasts. These Cys-gatekeeper kinases were sensitive to **ASDO2/6**-inhibition, but not AS-kinase inhibitor 3MB-PP1 and vice versa. These compounds, with AS-kinase inhibitors, have the potential to inhibit multiple AS-kinases independently with applications in systems level and translational kinase research as well as the rational design of type II kinase inhibitors targeting endogenous kinases.

**Overview of kinases and analog-sensitive kinase technology.** Eukaryotic protein kinases represent one of the largest protein families in the human genome with ~520 members and constituting about 2% of all human genes[1]. These enzymes catalyze the transfer of the γ-phosphate group from ATP to serine (Ser), threonine (Thr) or tyrosine residues on their substrate proteins or peptides. This post-translational modification is a critical and nearly

ubiquitous mode of intracellular signaling, with about 30% of all proteins being phosphorylated[2, 3]. It is, therefore, to no surprise that aberrant expression and activation of this class of proteins often leads to a variety of diseases with about 244 kinases mapping to disease loci and cancer amplicons[1, 4, 5].

One way to study kinase function and manage dysregulated kinases is through development of selective kinase inhibitors; however, due to the high degree of structural similarity, particularly in and around the ATP-binding pocket, it is often difficult to target an individual kinase with small-molecule inhibitors. One way to circumvent this issue is by employing a chemical-genetic strategy that takes advantage of a structurally conserved, mostly hydrophobic residue within the kinase active-site, termed the gatekeeper residue[6-8]. Mutation of this residue from a normally bulky residue to a smaller one, such as alanine (Ala) or glycine (Gly), engenders an analog-sensitive (AS) kinase containing a unique binding-site that can be exploited pharmacologically with bulky ATP-analogs such as 1NM-PP1[8], 3MB-PP1[8] and the recently developed staralogs[7] (Figure 1A-D). This 'bump-hole' method has been utilized for the study of at least 85 different kinases, yet despite this level of success there are still a number of kinases, coined 'intolerant' kinases, that do not tolerate gatekeeper mutations[8-11]. To expand the scope of AS-technology, researchers have identified second-site mutations, or suppressor of Gly gatekeeper mutations (*sogg*), that restore the kinase activity to some 'intolerant' AS-kinases (applied to at least 11 kinases so far) when the gatekeeper is Gly or Ala[8, 10]. In addition, electrophile-sensitive (ES)-kinases can be selectively inhibited by electrophilic, bulky PP1- or ATP-analogs (Figure 1E) (*e.g.* fluoromethylketobenzyl (FMKB)-PP1, acrylamido-anilinoquinazolines (AQZ) and 5'-vinylsulfonyl adenosine (VSA)) by targeting cysteine (Cys)-mutations within the gatekeeper or other positions vicinal to the ATP-binding pocket (*i.e.* in the Gly-rich loop (G-loop) or hinge region, Figure 1A)[9, 12-18]. However, these approaches appear to be highly specific to individual kinases and not widely applicable. This also limits the possibility to differentially inhibit two kinases with two orthogonal chemical genetic systems. Such a dual bio-orthogonal approach would be ideal for investigating kinase signaling crosstalk, synthetic lethality and many other areas of systems biology and translational research across a diverse set of kinase drug targets.

In this study, we report the development of a new chemical-genetic approach based on a Cys-gatekeeper mutation and non-covalent, type II mode of kinase inhibition that targets the inactive "DFG-out" kinase conformation (Figure 1C)[19]. We verified this approach with three divergent Ser/Thr-kinases: Greatwall kinase (GWL), Aurora-A kinase (AAK) and Cyclin-dependent kinase-1 (Cdk1). To demonstrate compound efficacy in cells, we measured phosphorylation of the physiologic GWL substrate $\alpha$-endosulfine (ENSA)[20] and cellular

proliferation, a phenotype functionally linked to GWL activity[21, 22], in wild type (WT)- and M110C (MC)-GWL expressing mouse embryonic fibroblasts (MEFs). **ASDO2** (**A**nalog-**S**ensitive "**D**FG-**O**ut" kinase inhibitor-**2**) specifically inhibited production of phospho-ENSA (pENSA) and attenuated proliferation in MC-GWL expressing MEFs, but not in WT-GWL expressing cells. Together, these data support expanding the AS-kinase inhibitor tool set with "DFG-out" targeting **ASDO** inhibitors, which in combination allow for the independent targeting of at least two distinct kinases (Figure 1C).

## RESULTS AND DISCUSSION

**Approaches utilized to expand analog-sensitive kinase technology.** The starting point of this study was an effort to develop an AS-version of microtubule-associated Ser/Thr kinase-like (MASTL) protein, commonly known as GWL, in order to investigate its mitotic functions and role in the cell cycle[23]. Over-expression and immunoprecipitation of FLAG-tagged GWL constructs provided a means to assess the activity of a cohort of GWL mutants (Figure 2A). This assay demonstrated that GWL is an 'intolerant' kinase evidenced by the inactivity of the *AS1* (M110G) and *AS2* (M110A) GWL kinase alleles (Figures 2B-C & S1A), and neither *sogg* mutations nor the ES-kinase approach were capable of generating an AS-GWL system (Figure S1A-G). In the first instance, we decided to make *sogg* mutations at the analogous positions in GWL, which included mutations that were previously successful for kinases such as Pto (L68I), APH(3')-IIIa (N268T) and GRK2 (S268V) (Figure S1G)[10]. Only one of these mutations, V61I-GWL, augmented GWL activity, evidenced by increased myelin basic protein phosphorylation, but did not restore activity of the double mutant V61I/M110A-GWL (Figure S1B). Bioinformatically, we identified sites in the G-loop consensus sequence (GxGxxG) that deviated from other AGC kinases, but reversion mutations in the G-loop, made to adopt the more common amino acids, had a meagre (S42G) to no (S42G/A45S) effect in combination with M110A-GWL (Figure S1, panels A & C)[24].

Initial efforts to identify *sogg* mutations for Ala-gatekeeper GWL were unsuccessful, and thus we hypothesized that other small amino acids, presumably preserving an expanded hydrophobic ATP-binding pocket, might be useful in combination with previously identified or new *sogg* mutations. To explore this idea further, the gatekeeper position was scanned with relatively small amino acids, which led to the discovery that MC-GWL-mediated phosphorylation of ENSA was only 4-fold less compared to WT (Figure 2B-C). Furthermore, through FACS analysis, we showed that RNAi-resistant MC-GWL, unlike M110A-GWL, was able to restore $G_1$, S and $G_2$/M cellular levels to the same extent as an siRNA-resistant WT rescuing plasmid in GWL depleted HeLa cells (Figures 2D-I & S2A-F). This result inspired the

generation of an ES-kinase system; however, the electrophilic inhibitors AG1-2 and FMKB-PP1 (Figures 1E, S1D-E & S2), which were validated against T338C-c-Src, did not robustly inhibit MC-GWL at 20 μM concentrations (Figure S1D-E)[9]. Of the other small gatekeeper mutations, M110T-GWL displayed ~8% activity compared to WT, and thus we made Cys mutations in an M110T-GWL mutant to mimic the cysteines in the hinge regions of EphB3 (C717) and EGFR (C797); M110T/G116C and M110T/D117C[13, 15]. In combination with a Thr gatekeeper, similar mutations in EphB1 (G703C) and c-Src (S345C), to name a few, are targetable using electrophilic quinazoline-based inhibitors (*e.g.* AQZ, Figure 1D), but due to the inactivity of these GWL double mutants, we were not able to apply them (Figure S1F-G)[13, 15].

**AD57-analogs specifically target MC-GWL *in vitro*.** Due to the inefficacy of known ES-kinase inhibitors against MC-GWL, we decided to generate novel inhibitors based on the structure of AD57[25] (Figures 3A & S3). We surmised that replacement of the urea linker with an acrylamide linker might allow selective targeting by a nucleophilic Cys-mutant[16]. AD57 also seemed like an ideal candidate as it selectively inhibited MC-GWL with an $IC_{50}$ value of 10.6 ± 0.4 μM and displayed no inhibitory activity against WT-GWL (Figure 3, panels B & D). **ASDO1**, the first in a series of AD57-analogs, was synthesized by coupling of an anilinopyrazolopyrimidine with a 3-benzoylacrylic acid chloride (Supplemental Schemes 1–3 & Figure 4A)[9, 26]. The resulting compound contained an acrylamide linker bridging the pyrazolopyrimidine moiety with a trifluoromethylbenzoyl group. This feature is similar to a trifluoromethylphenyl group, which is commonly incorporated into some type II kinase inhibitors[19, 27]. **ASDO1** (**A**nalog-**S**ensitive "**D**FG-**O**ut" kinase inhibitor-**1**) (Figure 3A) completely abrogated MC-GWL activity at a concentration of 20 μM without inhibiting WT-GWL (Figure S3). By assaying AD57 and **ASDO1** more rigorously using the immunoprecipitation-based kinase assay with ENSA as substrate, we were able to show that **ASDO1** exhibited a >60-fold improvement in potency against MC-GWL with an $IC_{50}$ value of 0.17 ± 0.09 μM and remained exquisitely selective for MC- vs. WT-GWL even up to concentrations as high as 100 μM (Figures 3C-D, 4A & S4A-B).

Structural studies using an AD57-c-Src co-crystal structure predicted that formal halogenation of the terminal phenyl ring with *ortho*-fluorine and *para*-chlorine substituents would fine-tune the selectivity profile of AD57. Indeed, these halogenated AD57-derivatives maintained inhibitory activity toward Ret, Raf, Src and S6K, reduced mTor inhibition and led to significantly less toxicity in a multiple endocrine neoplasia type 2 *Drosophila* model system[25]. In an attempt to fine-tune the selectivity of **ASDO1** toward MC- vs. WT-GWL, we synthesized halogenated

and bulkier, non-halogenated **ASDO**-derivatives by first incorporating acetophenones into the microwave-assisted aldol-condensation with glyoxylic acid to yield 3-benzoylacrylic acids[26], which after conversion to acid chlorides were later coupled with anilinopyrazolopyrimidines (Supplemental Schemes 2–3 & Figure 4A) to produce **ASDOs 2** – **6**.

**ASDO2** contains a trifluoromethylbenzoyl group with a *para*-fluoro substituent and demonstrated an ~3-fold improvement in potency, whereas the effect of the *para*-chloro substitution (**ASDO3**) was negligible (Figures 4A-C & S4C-E). We also explored the activity of bulkier derivatives in hopes of improving their selectivity for Cys-gatekeeper versus WT kinases. Unfortunately, replacing the central benzene ring with a naphthyl (**ASDO4,** Figures 4A & S4F) or 3-methylbenzyl group (**ASDO5,** Figures 4A & S4G) dramatically weakened inhibitory activity; however, the 2-methylphenyl substitution of the inner-benzene ring (**ASDO6**) in combination with the *para*-fluoro substitution seen in **ASDO2** resulted in a compound with inhibitory activity almost equal to that of the **ASDO1** precursor (Figures 4A, C-D & S4H-I). Interestingly, **DO1**, which lacks the pyrazolopyrimidine group, also displayed mono-specific targeting of MC-GWL vs. WT, but with attenuated inhibitory activity compared to **ASDO1** (Figures 4A, C & S4J-K), suggesting that the selectivity filter resulting in Cys-gatekeeper kinase specificity lies within the phenylbenzoylacrylamide scaffold and that the electrophilic olefin might be targeted by the Cys-gatekeeper residue. Furthermore, replacement of the trifluoromethyl group of **DO1** with a *para*-ethoxy substituent (**DO2**) completely ablated inhibitory activity toward both WT- and MC-GWL (Figures 4A, C & S4L-M). Interestingly, some type II kinase inhibitors (*e.g.* AD57 and Sorafenib[25, 27]) possess a trifluoromethyl substituent on their terminal phenyl ring, which occupies space in an allosteric hydrophobic pocket unique to the inactive kinase conformation[19, 28]. In contrast to **DO1**, the lack of inhibitory activity of **DO2** hints at the possibility that this series of compounds, like the parent compound AD57, might stabilize the inactive 'DFG-out' kinase conformation.

To establish the importance of the bulkier central ring of **ASDO6**, both **ASDO2** & **6** were subjected to a rigorous kinase inhibition-profiling screen (International Centre for Kinase Profiling, University of Dundee Kinase Profiling Express Screen). At 1 μM, **ASDO2** inhibited 15 of 50 kinases with inhibitory activity ≥ 60% (60–95%); however, as is the case with classical AS-kinase inhibitors, the increased bulk of the central benzene ring of **ASDO6** likely accounts for the decrease in number of WT-kinases targeted with inhibitory activity > 60% (62–84%); only 4 of 50 kinases. Note that the higher end of the inhibitory range decreased from 95% for **ASDO2** to 84% for **ASDO6**, providing further evidence that the bulkier **ASDO6**-derivative is more refractory toward WT-kinases (Figures 4E & S5A-B).

**ASDO6 targets AAK and Cdk1 bearing a Cys-gatekeeper.** Next, we sought to place **ASDO**-compounds within the pantheon of general AS-kinase inhibitors. To do this, we mutated the gatekeeper positions of AAK (L210) and *Xenopus laevis* Cdk1 (F80) to cysteines and subjected them to kinase assays with increasing concentrations of **ASDO2** or **6** (Figures 5A-F & S6A-C). Both kinases tolerated the Cys-gatekeeper mutation with improved (Cdk1) or a slight reduction (AAK) in kinase activity compared to WT (Figure S6B-C). Moreover, **ASDO2** inhibited L210C (LC)-AAK with the greatest potency, demonstrating an $IC_{50}$ value of 25 ± 2 nM (Figures 4A & 5, panels A & C) and was >70-fold selective in targeting LC-AAK vs. WT-AAK. Although less potent than **ASDO2**, **ASDO6** inhibition of LC-AAK ($IC_{50}$ = 1.7 ± 0.4 μM) and F80C-Cdk1 was about 10-fold more selective vs. their WT counterparts, respectively (Figure 5B-F).

**ASDO2 selectively inhibits MC-GWL in cells.** Although **ASDO6** inhibited less WT-kinases with inhibitory activity >60% (Figure 4E), **ASDO2** displayed greater potency and specificity in GWL and AAK kinase assays (Figure 4A). We, therefore, opted to assess the effects of **ASDO2** on cellular ENSA phosphorylation and proliferation in MEFs over-expressing WT- or MC-GWL. To establish cellular models for MC-GWL, we decided to employ MEFs containing loxP sites flanking exon 4 of the murine GWL gene[29]. Treatment of these cells with Cre-expressing adenovirus depleted GWL and allowed for reintroduction of human WT- or MC-GWL cDNA via lentiviral transduction (Figure 6A). After confirming the presence of human GWL by western analysis, both populations of WT- and MC-GWL expressing cells were treated with nocodazole (200 ng $mL^{-1}$) for 16–20 h (Figure 6B). The activity of mitotic kinases increases substantially during mitosis, making the nocodazole-mediated mitotic arrest (M-arrest) essential for visualizing the cellular inhibition of ENSA phosphorylation; inhibition of an inactive kinase would only reveal background level phosphorylation. After M-arrest and 4 h of 2 μM **ASDO2** or DMSO treatment, cells were harvested, lysed and probed with anti-pENSA antibody by western blot analysis, revealing mono-specific inhibition of MC-GWL-mediated ENSA phosphorylation (Figure 6C-D). Functionally, GWL has been linked to cellular proliferation in several studies, and thus serves as an ideal gauge for GWL activity[21, 22]. After 5 d of growth in the presence of inhibitor or DMSO, we observed that only cells depleted of endogenous WT-GWL and over-expressing MC-GWL had dramatically reduced proliferation in an **ASDO2** concentration-dependent manner, thus confirming the utility of **ASDO2** and possibly other **ASDO**-derivatives in cellular models (Figure 6E).

**ASDO2 displays relatively slow-on/slow-off binding/dissociation kinetics.** At this point, it was still unclear how **ASDO2/6** mono-specifically targeted MC- vs. WT-GWL (*i.e.* as a type

I or II inhibitor) and the presence of an electrophilic, doubly-activated olefin brings to question whether or not it interacts with MC-GWL in a reversible or irreversible manner. To further probe the mechanism of action of **ASDO2**, we investigated the reversibility of inhibition in comparison to a type I inhibitor (staurosporine) and the parent type II inhibitor AD57; note that type I inhibitors generally display fast-on/fast-off binding/dissociation kinetics, whereas type II inhibitors generally display slow-on/slow-off binding/dissociation kinetics[28, 30].

Initially, a washout experiment with MC-GWL supported the notion of an ES-kinase model system. Evidence for this is seen by the failure of **ASDO2**-treated (10 μM) MC-GWL to regain activity after 5 washes over a 4 h period compared to the quick reactivation of staurosporine (STP)-treated (50 μM) MC-GWL under the same conditions (Figure 7A-C). However, when we probed reactivation of MC-GWL after longer periods of time following washout of **ASDO2** and AD57 (*bona fide* type II inhibitor), it became evident that MC-GWL regained activity over time, but this reactivation period took longer due to a relatively slower off-rate (Figure 7D-E). Importantly, activity may not have rebounded to levels demonstrated by fully active MC-GWL (not treated with inhibitor) due to decomposition or destabilization of the protein over a 24 h period at 4 °C. Additionally, **ASDO2** rapidly inhibited MC-GWL activity, but with a slight difference in inhibitory activity between 20- and 60-minute compound pre-incubation periods (Figure 7F). Taken together, these data suggest that **ASDO2** shares similar slow-on/slow-off binding/dissociation kinetics as the type II inhibitor AD57, but could also be acting, to a small extent, in an irreversible manner as inhibition marginally improves over time (Figure 7F). Unfortunately, expression, purification and X-ray crystallography of full-length GWL has proven to be a major technical challenge; therefore, to pin down the exact binding mode of **ASDOs** in GWL, technical innovations in GWL structural biology are required[21].

**ASDO2/6 demonstrates a type II inhibitor binding mode with LC-AAK.** To cement the binding-mode of **ASDO2**/**6**, we solved X-ray co-crystal structures with LC-AAK: **ASDO2** (PDB: 6HJK) and **ASDO6** (PDB: 6HJJ)[31]. Both compounds were sandwiched between the N- and C-terminal lobes bound to the ATP-binding pocket, making the expected interactions with the backbone of the hinge region (Glu211 and Ala213) and stretching all the way to the Cα-helix, displacing it in comparison to the WT-AAK ADP-bound structure (PDB: 4CEG, Figure S7A-D). In both cases, the DFG-motif adopts an 'out' conformation (DFG-out), in which Phe275 points into the ATP-binding pocket, while the terminal aromatic groups of both compounds are buried in a hydrophobic pocket adjacent to the Cα-helix, indicative of type II kinase inhibitors (Figures 8A-B & S7D)[19]. In fact, overlay of the Asp274 residues of the 1NM-PP1- (type I, PDB: 4LGH) and **ASDO2/6-** (type II) bound structures illustrates another key difference between

type I and II inhibitors: The Asp residue flips 180$^{o}$ going from an active to an inactive conformation, allowing Phe to pack against **ASDO6** (Figures 8A-B & S7D)[19].

In the **ASDO6**-bound structure, Cys210 appears to form a weak, electrostatic interaction with the amide 'N', and the Ala273 backbone carbonyl forms a strong hydrogen bond with the same amide group, serving as a molecular clamp that also brings to light the importance of the cysteine residue (Figure 8C). Comparing the **ASDO2-** and **6-**bound structures also sheds light on the molecular level basis for the decrease in WT-kinase targeting by **ASDO6**; that **ASDO6** prefers binding to AAK only when the gatekeeper residue is a polar amino acid such as cysteine. Alignment of these structures shows that the methylphenyl ring of **ASDO6** twists in the direction of Cys210, bringing the amide closer to the Ala273 backbone; however, a water molecule fills the gap between **ASDO2**, Cys210 and the Ala273 backbone with all three elements forming a hydrogen-bonding network in the **ASDO2**-bound structure (Figures 8D-E & S7E).

As a result of placing the polar amide group of **ASDO6** in closer proximity to the gatekeeper residue, WT AAK, which harbors a hydrophobic Leu210 gatekeeper residue, would disfavor and destabilize the **ASDO6**-bound conformation (Figure 8F). Based on this hypothesis, other hydrophobic amino acids such as Ala should also attenuate **ASDO6** inhibitory activity and replacing cysteine with a less polar amino acid or with Gly, which lacks a side-chain, should abolish the molecular clamp mechanism and abrogate the **ASDO6**-GWL interaction as well. To test these hypotheses, we over-expressed, immunoprecipitated and treated FLAG-M110A-GWL with **ASDO6** up to a concentration of 10 μM. As predicted, **ASDO6** was not able to inhibit M110A-GWL, nor was 3MB-PP1 able to inhibit M110C-GWL *in vitro* (Figure 8G-H), suggesting the two systems work independently of each other and could be used to differentially inhibit other combinations of kinases based on gatekeeper mutations to either cysteine or Ala/Gly.

**Discussion and Conclusions**

AS-kinase technology has been employed to dissect the cellular functions of individual kinases across the kinase family, but there are still a number of kinases that remain averse to this methodology. In order to apply AS-kinase technology, mutations that engender sensitivity to pharmacologic inhibition must also maintain sufficient kinase activity so that the mutant kinase can recapitulate WT cellular function. There are many ways to broaden the scope of AS-kinase technology such as making *sogg* mutations that rescues the activity of 'intolerant' kinases when an Ala/Gly-gatekeeper mutation is present[10]. Also, over the years, a number of different AS- and ES-systems have been developed that incorporate amino acids other than Ala or Gly, *e.g.* Cys or Thr[9, 13, 17, 18], into the gatekeeper position, but so far, these systems have not been

widely utilized in the field; though some are still in their infancy. In terms of AS-systems that have proven fruitful, a number of new reagents such as staralogs[7] offer even greater specificity and potency, but to expand the scope of AS-kinase technology further we need not focus solely on engineering inhibitors against the active kinase conformation, but take full advantage of all modes of pharmacologic inhibition. To date, type II inhibitors have been left out of the loop when it comes to the development of chemogenomic technology, but this study is the first to provide an example of a chemical genetic system based on Cys-mutant kinase inhibition with a type II inhibitor; a system that we now refer to as the **ASDO** (**A**nalog-**S**ensitive '**D**FG-**O**ut')-kinase system (Figure 1B & C).

Furthermore, type I and II kinase inhibitors bind to disparate kinase conformations resulting in their catalytic inactivation[19, 27, 28]. Due to evolutionary pressure to preserve the catalytically active kinase conformation, type I inhibitors encounter a very similar ATP-binding pocket across the kinome, made exploitable through the 'bump-hole' method. The inactive kinase conformation is not bound by this evolutionary pressure and is more varied[28], which may have hampered previous attempts to generate a type II inhibitor-based **ASDO**-kinase system. Despite this variability, we systematically optimized the **ASDO**-scaffold to engender a small-molecule inhibitor that displays generality across at least three divergent Ser/Thr-kinases bearing a Cys-gatekeeper, so far, and **ASDO2/6** display bio-orthogonality versus WT kinases (Figures 1B & 4F). As this system not only appears to take advantage of steric complementarity, but also electrostatic interactions, to engender specificity toward Cys-gatekeeper kinases, we hypothesized that it may act independently of the canonical AS-kinase system, which is based solely on steric complementarity. This theory was validated *in vitro* and will likely change the landscape of future studies involving signaling pathways in cells and disease models. These multiple AS/ASDO-kinase systems along with the *Ele-Cys* and ES-kinase systems (systems that utilize electrophilic-quinazoline and 5'-electrophilic adenosine scaffolds) now provide an ensemble of chemical genetic tools to explore differential and independent kinase inhibition across at least two kinases in cells.

Lastly, this system exploits binding to and stabilizing the 'DFG-out' conformation of kinases and we demonstrated the tenability of isolating these **ASDO**-co-crystal structures using X-ray crystallography. To date, X-ray crystal structures of inactive kinase conformations across the kinome are sparse[19, 28], but the **ASDO** system could potentially be used to generate X-ray crystal structures of other inactive kinase conformations. This technology, therefore, has great potential to galvanize drug discovery efforts by providing new 'DFG-out' X-ray crystal structures that would assist with rational, *in silico* drug design efforts.

## METHODS

### Kinase Assays

Immunoprecipitation and radioactive kinase assays were carried out as published previously[21]. The catalytically dead mutant D174A-GWL was used as a control for inhibition. Aurora-A and xlCDK1 assays were carried out similarly to FLAG-GWL immunoprecipitation kinase assays. N-terminally tagged FLAG-Aurora-A[32] kinase and C-terminally tagged MYC-xlCDK1 were over-expressed in HEK 293T cells using the standard phosphate-mediated transfection method. Cdk1 assays were carried out by incubating HEK 293T lysates containing MYC-CDK1 with 4 μg of anti-c-Myc antibody and immunoprecipitation with 5 μl of Dynabeads™ Protein G (Thermofisher). To detect phosphorylated histones H1 (CDK1, Fig. S5A) and H3 (1μg/20μl reaction (AAK)) the standard kinase reaction was spiked with 0.075 MBq $\gamma$-$^{32}$P ATP (PerkinElmer) per 20 μl reaction. After the reaction was stopped with 5 μl 5X SDS loading buffer and boiled for 5 min at 95 °C, the mixture was resolved via SDS-PAGE (4-15% Criterion pre-cast gels (Bio-Rad Laboratories) or 13% v/v SDS-polyacrylamide gels). Staining with coomassie-blue revealed ~28 and 21 kDa bands respectively that were imaged by autoradiography. All concentration-dependent kinase assays were performed thrice and quantitation of activity (Phospho-signal(radioactive/densitometry):kinase loading ratio) using ImageJ (1.47v) of the average normalized % kinase activity ± S.D. was plotted using Prism 6.0. Non-linear regression using Prism 6.0 was used to calculate $IC_{50}$ values.

### Crystallization and Data Collection

Aurora-A (containing mutations L210C, C290A and C393A)[31] was co-crystallized with **ASDO2** and **ASDO6** by mixing purified protein (500 μM) with compound (**ASDO2**, 500 μM; **ASDO6**, 1mM) in a 1:1 protein:ligand ratio, before setting up sitting-drop vapor diffusion experiments at 22 °C; where 0.25 μl of complex was mixed with 0.25 μl of crystallization buffer and equilibrated against a well volume of 50 μl.

Crystals of Aurora-A bound to **ASDO2** were obtained in condition F6 of the PEGs II Suite (0.2 M ammonium sulfate, 0.1 M tri-sodium citrate pH 5.6, 25 % w/v polyethylene glycol 4000; Qiagen, USA) and bound to **ASDO6** in condition A1 of the JCSG+ Suite (0.2 M lithium sulfate, 0.1 M sodium acetate pH 4.5, 50 % w/v polyethylene glycol 400; Qiagen, USA). No additional cryo-protectant was added as the crystals were deemed already cryo-protected from their crystallization conditions. The crystals were cryo-cooled in liquid nitrogen and no ice formation was observed. Diffraction data to 2.4 Å (**ASDO2**) and 2.1 Å (**ASDO6**) resolution were collected at the Diamond Light Source [DLS, Didcot, UK] on beamline I04.

**Phasing, Model Building and Refinement**

All diffraction data were collected at 100K. Autoprocessed datasets were generated by automatic integration of the data using the software package XDS followed by processing using the Pointless and Scala programs from the CCP4 software suite. Phases were obtained by molecular replacement using Phaser with a high resolution structure of ADP-bound Aurora-A C290A,C393A (PDB: 4CEG) used as the search model. An iterative combination of manual building in Coot and refinement with Phenix.refine produced the final model: PDB codes 6HJK (ASDO2) and 6HJJ (ASDO6). The protein crystallized in spacegroups $P3_221$ (**ASDO2**) and $P6_122$ (**ASDO6**), with a single molecule comprising the asymmetric unit. Crystal data and structure refinement for **ASDO2/6** can be found in **Supplemental Tables 1** & **2**.

## SUPPORTING INFORMATION

Supplementary figures, tables and schemes, supplementary methods and characterization of synthetic compounds are supplied as supporting information. This material is available free of charge via the internet at http://pubs.acs.org.

## AUTHOR INFORMATION

The authors declare no competing financial interests.

## PDB ACCESSION NUMBER

6HJK: Aurora-A kinase (L210C, C290A and C393A) co-crystallized with ASDO2

6HJJ: Aurora-A kinase (L210C, C290A and C393A) co-crystallized with ASDO6

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## FIGURE LEGENDS

**Figure 1. AS- and ASDO-kinase systems**. **A**) Tertiary structure of the Staurosporine-bound GWL domain with key features highlighted (PDB: 5LOH). **B**) Chemical structures of 1NM-PP1 and ASDO2. **C**) Cartoon depicting a wild type kinase (top) with a bulky methionine gatekeeper residue. Mutating the gatekeeper residue to a smaller alanine (or glycine) residue generates an analog-sensitive (AS) kinase (middle). Mutating the gatekeeper residue to a cysteine generates an AS-kinase that is susceptible to inhibition by novel 'DFG-out' conformation-targeting inhibitors (**ASDOs; ASDO2** depicted here, bottom). The AS- and ASDO-kinase systems are orthogonal to wild type kinases and each other. **D**) A panel of reversible type I inhibitors that target alanine- or glycine-gatekeeper mutants. **E**) A panel of irreversible type I inhibitors that target cysteine mutations in the gatekeeper, hinge or G-loop regions. Structural features highlighted in **BLUE** complement the expanded hydrophobic pocket that is generated by mutation of the gatekeeper residue to alanine or glycine and features highlighted in **RED** represent cysteine-reactive, electrophilic warheads (fluoromethylketone, acrylamide and vinylsulfone).

**Figure 2. Systematic mutational analysis of the GWL gatekeeper position. A**) General workflow for the immunoprecipitation-mediated kinase assay. **B,C**) Systematic analysis of gatekeeper mutations in the GWL ATP-binding pocket. **D**) Rescue of RNAi-mediated GWL depletion by co-expression with siRNA-resistant WT- and MC-GWL constructs (pWTr and pMCr). **E-I**) FACS-cell cycle analysis of HeLa cells. Kinase assays were performed at least 3-times per mutation and quantitation using ImageJ (1.47v) (densitometry) of the average

normalized % kinase activity (pENSA:GWL ratio used as a readout of kinase activity) ± S.D. was performed and plotted using Prism 6.0.

**Figure 3. Establishing the inhibitory activity of AD57 and ASDO1 in the GWL kinase assay. A**) Chemical Structures of AD57 and **ASDO1**. Structural features highlighted in **Green** are linkers that bridge the PP1 moiety with the terminal trifluoromethylphenyl (**Red**) group. **B-C**) FLAG-tagged WT- and MC-GWL kinase assays in the presence of increasing concentrations of AD57 (**B**) or **ASDO1** (**C**). **D**) Kinase activity was quantitated using ImageJ (1.47v) (densitometry) and reported as the average normalized % kinase activity ± S.D.

**Figure 4. *In vitro* analysis of ASDO-derivatives. A**) The potency of synthetic AD57-derivatives (**ASDOs**), **DO1** and commercially available **DO2** was assessed using an *in vitro* immunoprecipitation kinase assay; na = not applicable and nd = not determined. The potencies of **ASDO2** (**B-C**) and **ASDO6** (**C-D**) were determined using FLAG-tagged WT- and MC-GWL kinase assays. **E**) **ASDO2** and **ASDO6** were screened at 1μM against a panel of 50 kinases, carefully selected to represent the kinome, using the Kinase Express Screen (International Centre for Kinase Profiling, University of Dundee).

**Figure 5. *In vitro* analysis of ASDO2/6 in FLAG-AAK and Myc-Cdk1 kinase assays.** The inhibitory activity of **ASDO2** (**A&C**) & **ASDO6** (**B-C**) against FLAG-tagged WT- and LC-AAK constructs was assessed using a radioactive kinase assay to detect $^{32}PO_4$-H3 substrate. **D-E)** The inhibitory activity of **ASDO6** against MYC-tagged WT and F80C-Cdk1 was assessed using a radioactive kinase assay for the detection of $^{32}PO_4$-H1. **C,F**) Kinase activity was quantitated using ImageJ (1.47v) (densitometry) and reported as the average normalized % kinase activity ± S.D.

**Figure 6. Cellular analysis of ASDO2 in WT- and MC-GWL expressing mouse embryonic fibroblasts (MEFs). A)** Expression of exogenous WT- and MC-GWL confirmed by western analysis. **B**) Strategy used to fully activate GWL in MEFs. Cells were treated with 200 ng/mL nocodazole for 16 – 20 h before harvesting for downstream applications. **C-D**) Mitotic cells were treated with 2μM **ASDO2** or DMSO for 4 h. ENSA, phospho-ENSA and tubulin levels in cell lysates were determined by western analysis. All experiments were repeated thrice and activity (pENSA:ENSA ratio) was quantitated using ImageJ (1.47v) (densitometry), normalized to DMSO control and reported as the average fold induction relative to DMSO control (without nocodazole) ± S.D. **E**) CellTiter Blue Proliferation assays.

**Figure 7. Comparing binding kinetics of ASDO2 to type I/II kinase inhibitors staurosporine (STP, type I) and AD57 (type II)**. FLAG-tagged MC-GWL was treated with DMSO (**A – F**), **ASDO2** (**A,C & D**), STP (**B,C**) or AD57 (**E**) for 20 minutes and then subjected to GWL kinase assay conditions. These beads were also subjected to several washes (buffer exchange) for the indicated amount of time before start of the kinase assay (**A – F**). **F**) Kinases assays were performed with FLAG-tagged MC-GWL, pre-incubated with DMSO or **ASDO2** for 20 or 60 m before start of the kinase reaction; reaction started by addition of ATP and ENSA. All experiments were repeated thrice and the average pENSA/FLAG(M110C-GWL) ratio was quantitated using ImageJ (1.47v) (densitometry) and normalized to DMSO control (**C**).

**Figure 8. X-ray co-crystal structures of ASDO2 and ASDO6 with LC Aurora-A kinase. A**) Alignment of **ASDO6 (6HJJ)**, ADP (4CEG) and 1NM-PP1 (4LGH) bound structures reveals the orientation of their respective catalytic-motif aspartic acid residues. **B**) A zoomed-in view of panel (**A**). **C**) **ASDO6** abuts the gatekeeper cysteine residue forming a weak electrostatic interaction with the thiol hydrogen of cysteine and a stronger hydrogen-bonding interaction with the backbone of Ala273, serving to clamp **ASDO6** within the nucleotide binding-site proximal to the gatekeeper position. **D**) Comparison of the **ASDO2**- and **ASDO6**-bound LC-AAK structures. **E**) A zoomed-in view of panel (**D**). **F**) Superimposition of the **ASDO6**- and ADP-bound structures reveals a potential conflict between the **ASDO6**-amide and the hydrophobic L210 gatekeeper residue. **G,H**) **ASDO6** (**G**) and 3MB-PP1 (**H**) inhibitory activity was assessed against M110A-GWL and MC-GWL respectively using the immunoprecipitation kinase assay.